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Purification and Characterization of 4-Hydroxyphenylpyruvate Dioxygenase from Maize

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Abstract: The proposed target enzyme for benzoylcyclohexanedione herbicides, 4-hydroxyphenylpyruvate dioxygenase (HPPD) was purified from etiolated maize seedlings with a purification factor of 105. Enzyme activity was measured by detection of carbon dioxide formed from radiolabelled substrate. The enzyme has a pH optimum of 7·3 and an apparent molecular mass of 43 kDa, similar to that of the mammalian liver enzyme. Activity needs the presence of a reducing system glutathione/dichlorophenol indophenol or ascorbate and catalase. Surprisingly, a commercial catalase preparation of low specific activity—generally used for the enzyme assay—showed HPPD activity which was separable from the catalase activity on a gel filtration column. According to kinetic studies with purified maize HPPD, experimental herbicides from the family mentioned were strong competitive inhibitors of the plant enzyme in nanomolar range with K_i values of 5 and 15 nM for 2-(2-nitro-4-chlorobenzoyl)-5-(2-methoxyethyl) cyclohexane- 1,3-dione and 2-(2-chloro-4-methanesulfonylbenzoyl)- cyclohexane-1,3-dione (SC-0051; sulcotrione), respectively.

Key words: liver catalase, maize, enzyme purification, herbicide, benzoylcyclohexanediones, 4-hydroxyphenylpyruvate dioxygenase E.C. 1.13.11.27, enzyme kinetics

1 INTRODUCTION

Benzoylcyclohexanediones (triketones) represent a relatively new group of bleaching herbicides patented in 1985.[1] According to the first investigations, these herbicides decreased the pigment content of different plants similarly to known bleaching herbicides such as norflurazon.[2,3] It was assumed that these herbicides are phytoene desaturase inhibitors. However, this hypothesis failed because these herbicides were not inhibitory *in vitro* in well-established phytoene desaturase systems.[4] A representative compound of this family, 2-(2-nitro-4-trifluoromethylbenzoyl)-cyclohexane-1,3-dione (NTBC) was reported to be an effective inhibitor of human liver 4-hydroxyphenylpyruvate dioxygenase (HPPD, E.C. 1.13.11.27) and was used to treat hereditary tyrosinemia type I *via* decreasing formation of toxic tyrosine catabolites.[5] The enzyme HPPD is well known in different organisms[6] and catalyses the starting reaction of plastoquinone and tocopherol biosynthesis in plants (Fig. 1).[7] Later on it was found that homogentisic acid, the product of HPPD-catalysed reaction, reversed the toxic

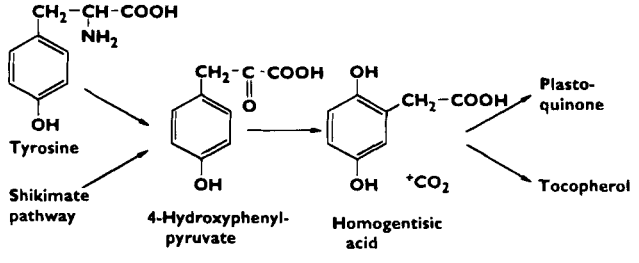

Fig. 1. Pathway of α-tocopherol and plastoquinone biosynthesis involving 4-hydroxyphenylpyruvate dioxygenase as a starting enzyme in plants.

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effects of SC-0051 herbicide on *Lemna gibba* L. This herbicide decreased the incorporation of label from tyrosine to lipophilic metabolites in wheat and, according to preliminary data, SC-0051 was a strong inhibitor of the maize enzyme.[8] In other experiments NTBC treatment elevated the tyrosine levels of different plants, and decreased the plastoquinone concentration in *Ipomoea hederacea* L. In preliminary studies with a crude enzyme fraction from maize, the herbicide was a competitive inhibitor.[9] It was suggested that the primary target of this herbicide class is HPPD, causing decreased tocopherol and plastoquinone levels, thereby indirectly inhibiting phytoene desaturation.[8,9] The relationship between quinone biosynthesis and phytoene desaturation, however, has not yet been convincingly demonstrated. It is known only that some quinones can substitute for oxygen as electron acceptor in the phytoene desaturation reaction *in vitro*.[10]

The objective of this study was to purify HPPD enzyme of maize for a clear demonstration and evaluation of its inhibition by benzoylcyclohexanedione herbicides. Because the enzyme has not yet been purified from plants, our preparation should make possible a thorough characterisation of the plant enzyme and comparison with the enzyme from other organisms.

2 MATERIALS AND METHODS

2.1 Preparation of substrate

Carboxyl-labelled 4-hydroxyphenyl[1-^{14}C]pyruvic acid was synthesised from L-[1-^{14}C]-tyrosine (NEN Research Products, Boston, MA, USA) according to literature methods.[11] A 20-μl portion of L-[1-^{14}C]-tyrosine (74 kBq, specific activity 2·0 GBq mmol^{-1}) dissolved in hydrochloric acid (1 M) was neutralized with sodium hydrogen carbonate solution (0·5 M; 25 μl) and diluted with phosphate buffer (0·1 M; pH 6·5; 130 μl). Then 1000 units of bovine liver catalase (spec. activity 38 000 U mg^{-1}, Sigma, St. Louis, USA) and 1 mg crude L-amino acid oxidase (from *Crotalus adamanteus* L., Sigma) were added. The mixture was kept on a shaker in an open vial for 80 min. The yellowish solution was loaded onto a column of 500 μl Dowex 50W X8 (Sigma) resin equilibrated with 1 M hydrochloric acid. The product containing 80–90% of the radioactivity was eluted with hydrochloric acid (0·1 M; 2·0 ml). The specific activity was adjusted with a 10 mM solution of non-labelled 4-hydroxyphenylpyruvic acid (Sigma) and the solution was stored at −20°C.

2.2 Assay of 4-hydroxyphenylpyruvate dioxygenase activity

The HPPD activity was measured by a radioactive method using ^{14}C-carboxyl-labelled substrate.[12] In this reaction, the 4-hydroxyphenylpyruvate substrate reacts to homogentisate and carbon dioxide (Fig. 1). Due to the carboxyl label of the substrate, the carbon dioxide formed was labelled and trapped with appropriate alkaline solution and counted for radioactivity.

The assay was performed in 20-ml plastic scintillation vials sealed with a rubber stopper through which two 11-mm diameter filter paper discs were placed on a hook of stainless-steel wire. The filter paper discs were soaked with methanolic bezethonium hydroxide solution (1 M; 25 μl; Sigma).

The standard 1·0 ml reaction mixture contained in 0·1 M phosphate buffer (pH 7·3), 50 mM ascorbate, 416·7 Bq 4-hydroxyphenyl[1-^{14}C]pyruvic acid with appropriate specific activity, 1000 units bovine liver catalase (spec. activity 38 000 U mg^{-1}) and enzyme enough to convert approximately 10% of the substrate during the time of assay. Prior to initiation with enzyme, the mixture was kept on ice. The vial was sealed, the enzyme solution was injected through the septum into the reaction mixture, and at zero time the vial was placed into a 30°C water bath for 30 min. The reaction was stopped by addition of sulfuric acid (1 M; 0·5 ml). After 30 min post-incubation, the filter paper discs were transferred into a scintillation vial containing methanol (0·4 ml). The vial was shaken for 10 min followed by adding Ultima Gold scintillation cocktail (3·6 ml; Packard Instruments, Groningen, Netherlands) and the radioactivity was measured in a Rackbeta II liquid scintillation counter (LKB, Turku, Finland).

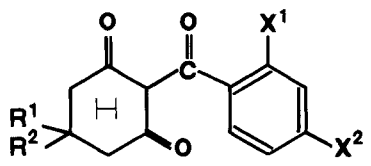
2.3 Plant material

Seeds of maize (*Zea mays* L.) hybrid SIL anjou 18 (Saaten-Union GmbH, Hannover, Germany) were washed with tap water several times until the seed-dressing had been removed from the surface of seeds. The seeds were soaked in tap water for one day and then germinated between wet vermiculite and filter paper in the dark at 22°C for four days.

2.4 Enzyme purification

The purification procedure was performed at 4°C in series without freezing and storing the intermediate fractions.

Crude maize homogenate was prepared according to the method of Schulz *et al.*[8] The homogenisation buffer contained 2 g litre^{-1} polyvinylpolypyrrolidone (Sigma), 0·2 g litre^{-1} glutathione (Sigma) and 0·14 M potassium chloride in 0·02 M phosphate buffer (pH 7·0). About 150 g of etiolated seedlings and 100 of buffer were mixed at 11 000 rev min^{-1} for 2 × 10 s in a Sorvall homogenizer (Sorvall Inc., Newtown, Conn., USA). The homogenate was filtered through two layers of miracloth and the filtrate was centrifuged for 30 min at



Compound	R^1	R^2	X^1	X^2
CH-309	CH_3	CH_3	NO_2	Cl
251541	H	$CH_3OC_2H_4$	NO_2	Cl
251665	H	$CH_3OC_2H_4$	NO_2	H
SC-0051	H	H	Cl	SO_2CH_3

Fig. 2. Chemical structures of benzoylcyclohexanedione herbicides used in inhibition experiments.

12 000*g*. The supernatant was fractionated by ammonium sulfate precipitation at 0°C. The proteins precipitated between 20 and 40% ammonium sulfate saturation were collected and redissolved in Tris-HCl buffer (0·05 M; pH 8) and desalted on PD-10 columns (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer.

The desalted protein solution was applied to a 2 × 10 cm column of DEAE Sepharose CL-6B (Pharmacia) previously equilibrated with Tris-HCl buffer (0·05 M; pH 8·2, starting buffer). The column was washed first with 200 ml of the starting buffer containing potassium chloride (0·05 M) at a flow rate of 100 ml h^{-1}, then developed with 200 ml linear gradient of potassium chloride from 0·05 to 0·2 M in the starting buffer. The fractions (about 100 ml) from the gradient containing the activity were collected and dialysed against 0·05 M sodium acetate-acetic acid buffer (pH 5·2).

A 1 × 10 cm column of S-Sepharose (Sigma) was equilibrated with 0·05 M sodium acetate-acetic acid buffer (pH 5·2). The dialysed protein solution was loaded to this column at a flow rate of 50 ml h^{-1}. The column was washed with the same buffer (approximately 30 ml) until a stable base-line was detected on the UV detector. Proteins were eluted with the starting buffer containing sodium chloride (0·05 M). The active fractions were collected and dialysed against phosphate buffer (0·025 M; pH 6·5). This dialysed solution was snap-frozen in small portions in liquid nitrogen, stored at −20°C without detectable loss of activity for months or used for other experiments.

When the active fractions from the S-Sepharose column were processed further, before final dialysis, 15 ml of the solution was concentrated in a Centriprep concentrator 3 (Amicon, Beverly, MA USA) to 1·5 ml. The concentrate was injected onto a Superose 12 10/30 gel filtration column attached to an FPLC system (Pharmacia). The column was previously equilibrated and eluted with phosphate buffer (0·1 M; pH 6·5) containing sodium chloride (0·2 M) at a flow rate of 0·3 ml min^{-1}. The UV detector showed one small and one major protein peak eluted at 14 and 15·2 ml, respectively and some minor impurities eluted after the major peak. The molecular mass of the major protein that contained the activity was 43 kDa. The FPLC fraction was not used for the enzyme studies.

2.5 Assay of catalase activity

Bovine liver catalase with specific activity of 1540 units mg^{-1} (Sigma, Lot 23H7035) had a considerable HPPD activity (a Sigma catalase unit is equivalent to 1 μmol min^{-1} mg^{-1} activity). Purified preparations (e.g. bovine liver catalase, 38 000 units mg^{-1}; Sigma), even at higher concentrations, were free of this contamination and therefore used routinely in the assays.

The catalase activity of commercial catalases and plant extracts was determined spectrophotometrically detecting the loss of hydrogen peroxide substrate at 240 nm in phosphate buffer (0·1 M; pH 7). Hydrogen peroxide concentration was about 10 mM.

2.6 Gel electrophoresis

The purification process was followed by SDS-polyacrylamide gel electrophoresis[13] on conventional gels with 1 mm thickness and 12·5% T. The protein samples as well as molecular weight markers (Pharmacia) were treated with mercaptoethanol. The developed gels were stained with Coomassie Brilliant Blue R-250.

2.7 Herbicides used for inhibition experiments

Experimental herbicides (Fig. 2) 251541 [2-(2-nitro-4-chlorobenzoyl)-5(2-methoxyethyl)cycohexane-1,3-dione], 251665 [2-(2-nitrobenzoyl)-5(2-methoxyethyl)-cyclohexane-1,3-dione] and CH-309 [2-(2-nitro-4-chlorobenzoyl)-5,5-dimethylcyclohexane-1,3-dione] were supplied by Nippon Soda (Odawara, Japan). SC-0051 [sulcotrione; 2-(2-chloro-4-methanesulfonylbenzoyl) cyclohexane-1,3-dione] was from Zeneca (Berkshire, UK).

2.8 Kinetic measurements

Substrate and inhibitor kinetics were measured in the substrate concentration range of 2·5–20 μM with the standard assay, in three replicates. The data were corrected for the non-enzymic reaction determined for each substrate concentration. Inhibitors were dissolved in dimethyl sulfoxide and further diluted with the assay buffer. The reaction mixture contained less than 0·02 ml $litre^{-1}$ solvent of the stock solution. Data were analysed

by linear regression of Lineweaver–Burk plots and showed good linearity.

3 RESULTS

3.1 HPPD activity of a commercial liver catalase enzyme

In an early stage of the experiments it turned out that the commercial catalase preparation used for the assay had high HPPD activity. The radioactivity measured definitely originated from the catalase preparation, because it correlated proportionally with the enzyme amount added to the assay; a boiled enzyme did not produce activity, and the substrate on its own or together with the reducing system formed only very low amounts of the labelled product. This finding was unexpected and thus we tried to characterise this activity. The 7·3 pH optimum in 0·1 M phosphate buffer and the 30 μM K_M at the pH optimum were similar to those reported for different liver HPPD enzyme[11,12] and the herbicide CH-309 strongly inhibited the activity. This 'foreign' activity was not negligable, since generally 1000 units of catalase are included into the HPPD assay. In our system, at low substrate concentrations, this amount caused more than 50% conversion. This activity was also present in another vial from the same Sigma catalase preparation having the same lot number (data not shown), but was lower in a different, higher specific activity enzyme sample. Only the purest catalase enzyme with 38 000 units mg^{-1} specific activity did not show a HPPD reaction, even with an extremely high protein amount (100 000 units of catalase) added to the assay.

The HPPD activity of the catalase preparation may derive from a side activity of the catalase enzyme existing in early stages of purification but not in the pure product or, alternatively, may be an impurity of the crude enzyme. This latter assumption was corroborated by gel filtration experiments since the HPPD and the catalase activities of the catalase preparation could be separated on a Superose 12 column (Fig. 3). The peaks of the two activities corresponded to the appropriate molecular mass of catalase (240 kDa) and liver HPPD (89 kDa) enzymes. The SDS-PAGE analysis of a crude catalase showed a prominent band at the subunit molecular mass of the liver HPPD (43 kDa, Fig. 4) together with a number of other impurities which were not present in the commercial purified catalase.

3.2 Purification of the maize HPPD enzyme

The homogenisation and ammonium sulfate precipitation as described[8] was followed by desalting, DEAE Sepharose column, dialysis, S-Sepharose column and a final dialysis against phosphate buffer in which the

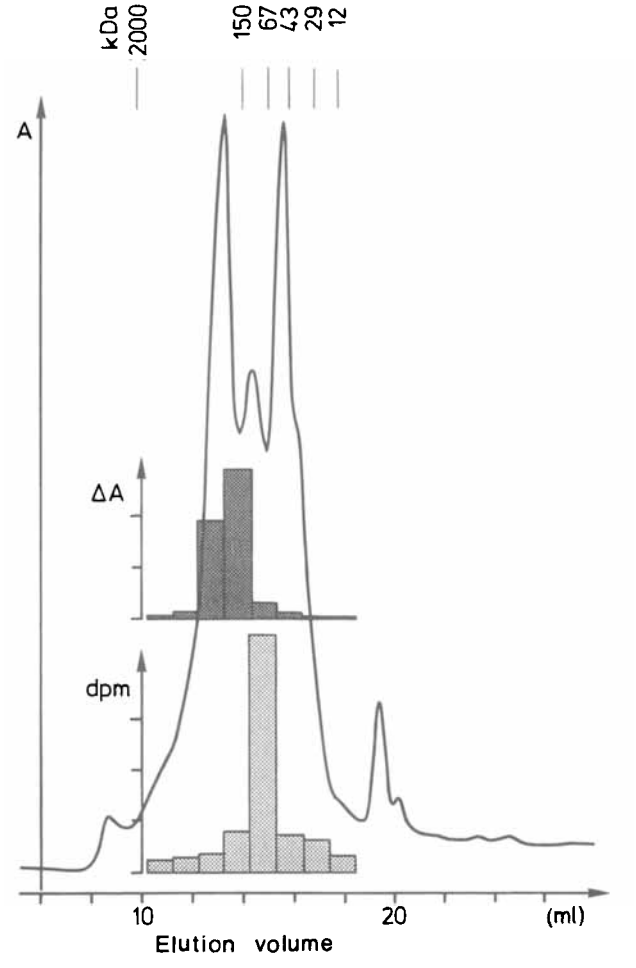


Fig. 3. Gel filtration chromatogram of commercial bovine liver catalase (specific activity 1540 units mg^{-1}) on a column of Superose 12 10/30 attached to an FPLC system. Sample concentration 10 mg ml^{-1}, injected volume 0·2 ml, eluent 0·2 M sodium chloride in 0·1 M phosphate buffer (pH 6·5), flow rate 0·3 ml min^{-1}. Proteins were detected on a UV detector. Fractions were assayed for catalase activity (upper inset, decrease of absorbance at 240 nm) and HPPD activity (lower inset, radioactivity measured in the assay). The marks on the top correspond to the elution volumes of standards with molecular mass given and determined under the same conditions.

enzyme was stored. This procedure started with 150 g of etiolated maize seedlings, enough to finish the whole process in one sequence, and provided sufficient enzyme for about 250 assays. The purification factor was 105-fold based on the desalted sample (Table 1). An unknown inhibitory compound substantially decreased the measurable specific activity in the crude homogenate and the precipitate. This inhibitor was a low molecular mass, probably non-protein material, because the boiled crude extract was still inhibitory (data not shown), and the desalting G-25-type column separated it from the proteins.

The DEAE column could bind the enzyme at a relatively high pH value (pH 8·2) and the enzyme tolerated it. The 64% recovery data presented in Table 1 was

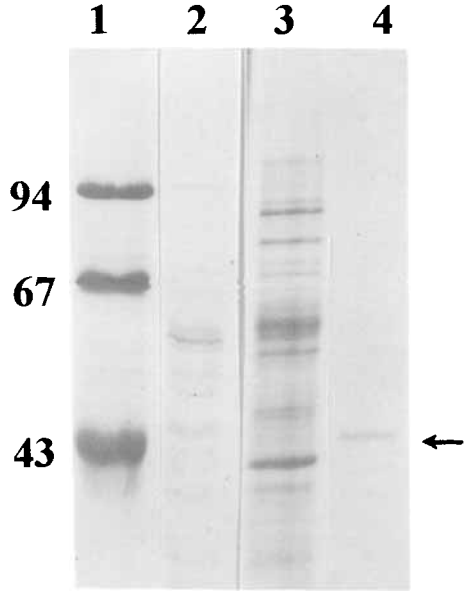


Fig. 4. Illustration of the purification of HPPD from maize by SDS-PAGE. Lane (1) protein standards with molecular mass of 94, 67 and 43 kDa, respectively; (2) commercial low specific activity catalase with HPPD activity; (3) active fraction from DEAE Sepharose column; (4) high specific activity fraction from S-Sepharose column.

sometimes even higher, depending mainly on both the protein amount loaded to the column and the flow rate. A strong cation exchanger, S-Sepharose, was used for the second column. To ensure an efficient purification, the pH of this column step had to be controlled precisely at 5·2. At pH 5·4, for example, only one-third of the total activity bound to the column. The main reason of the poor (about 20%) yield of this column was a major impurity that eluted at about the same ionic strength as the purified enzyme and had an apparent molecular mass about 60 kDa. The first fractions of the S-Sepharose column did not contain this 60 kDa protein and showed high specific activity. The concentration of this impurity, however, increased in the subsequent fractions, thereby decreasing the specific activity. About 30% of the total HPPD activity was eluted in these latter, low-specific-activity fractions which were not included into the total yield of this purification step. According to SDS-PAGE runs, the combined fractions of the S-Sepharose column contained one dominant protein band at 43 kDa. This fraction was basically free of the impurity mentioned above. The purification process is illustrated in Fig. 4.

In some cases, the pure S-column fractions were concentrated on Centriprep membrane concentrators and analysed by Superose 12 gel filtration column. The yield of the concentration step was not better than 60%, due mostly to the protein precipitation during this process. Again the chromatogram showed one major protein peak. The maximum of the HPPD activity was found in the fraction which contained this dominant protein and at the same elution volume which was detected for a 43 kDa standard (chromatogram not documented). Consequently, both electrophoresis and gel filtration yielded 43 kDa as molecular mass of the plant enzyme.

3.3 Characterisation of the purified maize HPPD enzyme

The characterisation as well as the kinetic experiments were done with the S-column fractions dialysed against 25 mM phosphate buffer (pH 6·5). This diluted enzyme solution (protein concentration was below 50 $\mu g\ ml^{-1}$) was stored for months in a freezer without loss of activity. After thawing, the enzyme was never frozen and stored again. The thawed enzyme was stable in ice about for a day.

The pH optimum of the enzyme was 7·3 in 0·1 M phosphate buffer (Fig. 5). The liver enzyme has the same pH optimum but that is active in a wider pH range.[12] The detectable activity increased twofold when the incubation temperature was raised from 23° to the optimum 30°C. Shaking of the reaction vessel only slightly influenced the reaction rate, indicating that the solution contained sufficient oxygen.

The HPPD enzyme from other organisms requires the presence of a reducing component. The plant enzyme also was active only if one of the reducing components, either glutathione/dichlorophenol imdophenol

TABLE 1
Purification of 4-Hydroxyphenylpyruvate Dioxygenase from Etiolated Maize Seedlings

Purification step	*Total protein (mg)*	*Total activity (%)*	*Specific activity[a] ($nmol\ h^{-1}\ mg^{-1}$)*	*Purification factor*
Crude extract	1660	(3·6)[b]		
Ammonium sulfate precipitate	725	(41)		
PD-20 desalting column	690	100	0·67	1
DEAE Sepharose column	40	64	7·37	11
S-Sepharose column	0·8	12·8	70·3	105

[a] Enzyme activity was determined at 1 μM substrate concentrations in the standard assay.
[b] The maximum total activity was found in the desalted sample since the plant extract contained an unknown inhibitory material.

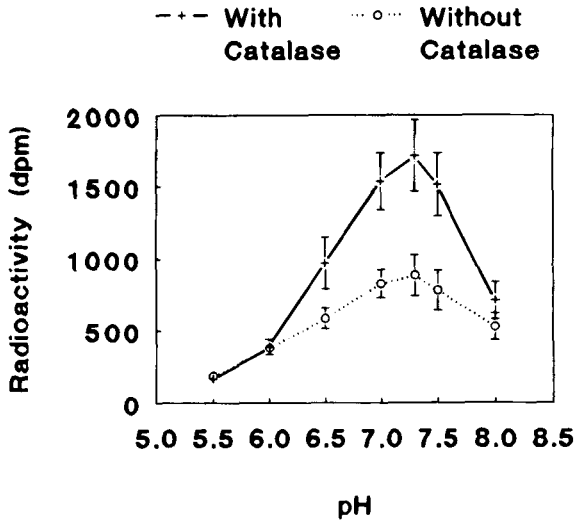


Fig. 5. Determination of pH optimum of purified 4-hydroxyphenylpyruvate dioxygenase (HPPD) from maize seedlings in 0·1 M phosphate buffer with or without catalase. Substrate concentration 1 μM, total radioactivity 25000 dpm/assay.

or ascorbate, was present in the reaction mixture. The maize enzyme was twice as active in the presence of ascorbate as with the optimum concentration of the first reducing system.

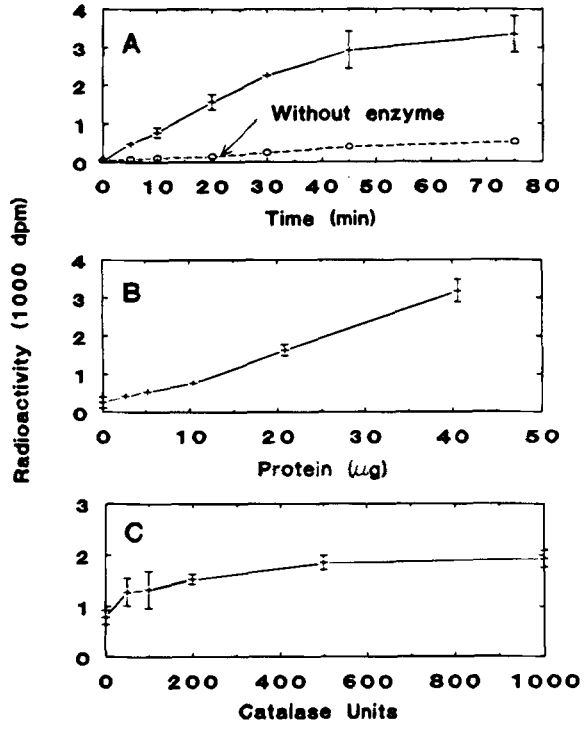


Fig. 6. Influence of (A) reaction time (B) protein amount and (C) catalase units used in the assay on HPPD activity of the purified maize enzyme as detected by formation of radioactive carbon dioxide. Substrate concentration 1 μM, total radioactivity 25000 dpm, reaction time in B and C 30 min, catalase units in A and B 1000.

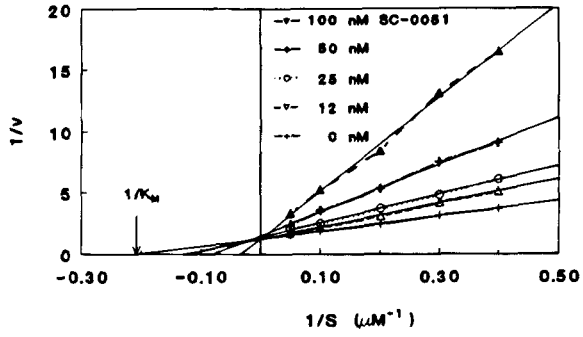


Fig. 7. Lineweaver-Burk double-reciprocal plot of kinetic data measured with purified HPPD of maize and SC-0051 herbicide as inhibitor. The plot is characteristic for competitive inhibitors.

The enzyme reaction was linear in the time range of 30 min (Fig. 6A). As expected, increasing the protein concentration—above a concentration limit—also proportionally increased the reaction rate (Fig. 6B). The HPPD activity was significantly enhanced with pure catalase present in the assay, reaching a maximum above 500 units (Fig. 6C). Probably, the enzyme forms hydrogen peroxide in a side reaction that is quenched by catalase, keeping the enzyme active longer. (It was found that hydrogen peroxide could chemically oxidise the substrate in a very rapid decarboxylation reaction.)

3.4 Kinetic measurements

The substrate kinetics of the purified enzyme was measured under our optimised conditions. The Lineweaver–Burk plot was linear in the substrate concentrations above 2 μM. Below this limit, the $1/v$ data calculated were generally lower than had been expected from the linearity (data not shown). The discrepancy was attributed to decomposition of the substrate during or before the assay. This chemical process has been reported to be quite rapid above pH 7, thus at the pH optimum of HPPD activity.[14] The K_M value determined from the linear concentration range was 5 μM (Fig. 7). A value of

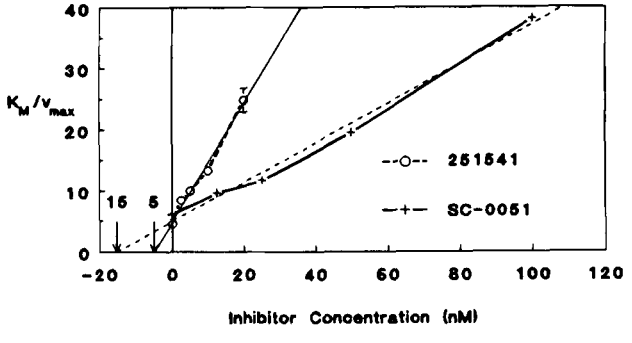


Fig. 8. Determination of inhibitor constant K_i of herbicides 251541 and SC-0051 from the slopes of double-reciprocal plots of kinetic data as presented by Fig. 7 for SC-0051. The inhibitor constants, x intercepts, are 5 nM for 251541 and 30 nM for SC-0051.

30 to 40 μM has been reported for the corresponding liver enzyme.[11,12]

The benzoylcyclohexanedione herbicides are strong inhibitors of the purified maize enzyme. The I_{50} values determined at 1 μM substrate concentration under standard assay conditions were found to be 3, 7, 11 and 23 nM for CH-309, 251541, SC-0051 and 251665, respectively. For herbicides 251541 and SC-0051, complete inhibitor kinetic measurements were performed. The Linewaver–Burk plot for the herbicide SC-0051 is shown in Fig. 7, while the determination of the inhibitor constants for both herbicides is illustrated by Fig. 8. These kinetic data show that both herbicides are competitive inhibitors of the HPPD enzyme. The inhibitor constants K_i are 5 and 15 nM for 251541 and SC-0051, respectively.

4 DISCUSSION

A novel finding of this investigation is that the commercial Sigma bovine liver catalase preparation is contaminated with HPPD activity, although previously routinely used for HPPD assay. This contamination depends strongly on specific activity, i.e. the purity of the catalase preparation. The highest specific activity catalase available did not have any measurable HPPD activity. Catalase and HPPD activities were separable on a gel filtration column, so the latter activity simply contaminated the crude catalase.

The purification described here for HPPD from maize is the first documented purification for a plant HPPD enzyme. The specific activity increased by a factor of 105, but an additional two-fold purification may be assumed from the decrease of the total protein in the fractions containing the HPPD activity in the precipitation and desalting steps. Because some inhibitory material was present in these first steps and was completely eliminated only by desalting, it was not possible to calculate the exact enrichment values for these two steps.

The purified enzyme preparation contained one major protein at 43 kDa according to SDS-PAGE as well as gel filtration. This molecular mass is equivalent to the subunit molecular mass of the liver enzyme from different sources. It is not clear whether or not the native plant enzyme is composed of two or more subunits as was described for the liver enzyme[11,12] but definitely a monomer exists in the later fractions, where the gel filtration experiments gave the same apparent molecular mass as SDS-gel electrophoresis.

The purified maize enzyme was used under optimised assay conditions in kinetic experiments to obtain clear inhibition data for different benzoylcyclohexanediones. This measurement is complicated by the instability and the keto-enol tautomerism of the substrate at the pH of the assay.[14] Most probably for this reason, the double reciprocal plot of substrate kinetic data was linear only above 2 μM. The chemical decomposition of the substrate also led to evolution of labelled carbon dioxide. In spite of determination of the non-enzymic reaction at each substrate concentrations, the kinetic data still were not linear at the concentration below the limit mentioned. Determination of kinetic data over a wider concentration range would be possible only by measuring homogentisic acid, the other product of the reaction, by, e.g. HPLC. This method is more complicated, a time- and cost-consuming process for the hundreds of assays needed for kinetic experiments, rather than the simple, very sensitive and generally used radioactive assay. The kinetic data published here were calculated from the linear part of the double reciprocal plots and the 2–20 μM concentration range provided reliable data yielding a K_M of 5 μM. The K_M value for the liver enzymes from various sources is in the range of 30–40 μM.

The benzoylcyclohexanediones investigated were strong inhibitors of the purified maize enzyme. The low nanomolar inhibitor constants give evidence that the primary target site of these herbicides is indeed the HPPD enzyme.[8,9,15] When first published, this suggestion was based on in-vivo experiments and in-vitro inhibition data (the I_{50} of SC-0051 was found to be 45 μM on maize enzyme, but substrate concentration and purification were not reported).[8] In another study, a similar I_{50} value was published for NTBC for a impure maize enzyme preparation and a competitive inhibition mechanism was assumed without giving details of the kinetic experiments and data.[9] A competitive mechanism has been verified by the kinetic data presented in this paper using a pure preparation. This inhibitory type is generally operative when a structural similarity exists between the substrate and the inhibitor molecules as has been discussed earlier for HPPD and triketones.[9] Because of this similarity it is possible that the enzyme might also metabolise the inhibitor molecules at least in a slow reaction.

This novel herbicide mechanism involves strong bleaching symptoms on the whole-plant level. The plants treated with a cyclohexanedione have extremely low chlorophyll and carotenoid contents. However, the exact relationship between the HPPD inhibition and the bleaching process is not known. According to preliminary data, NTBC decreased the plastoquinone level of a sensitive weed but this effect was also detected in the case of the phytoene desaturase inhibitor fluorochloridone.[9] Interestingly, both NTBC and fluorochloridone also increased the aromatic amino acid levels of this plant.[9] Apparently, phytoene desaturation and quinone biosynthesis are closely connected in plants, and it was suggested that a quinone functions as a redox component although conclusive experimental data have not been reported.[10,16,17] It is known

that different model quinones were able to substitute for oxygen in phytoene desaturation *in vitro*, but a clear understanding of this problem needs further studies.

A crucial question of herbicide biochemistry is selectivity. It has not been shown whether or not these herbicides are stronger inhibitors of HPPD from more herbicide-sensitive plants or, alternatively, metabolism is responsible for the herbicide tolerance of maize. Clarification of this question is possible only after comparison of metabolic and kinetic data obtained from different plants.

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